



## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

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<b>(54) Title:</b> PREPARATION OF BIOLOGICAL MATERIAL FOR IMPLANTS  <b>(57) Abstract</b>  A method for preparation of biological material for a medical device. Samples of intact or acellular tissue of biological material are treated in vitro with (i) a water-soluble carbodiimide of the formula $R_1-N=C=N-R_2$ , wherein $R_1$ and $R_2$ are independently selected from alkyl and alkylamino groups, in combination with (ii) an agent that forms a stable activated ester with said carbodiimide. The agent to stabilize the activated esters is preferably N-hydroxysuccinimide or N-hydroxysulfosuccinimide. The preferred carbodiimide is 1-ethyl-3-(dimethylaminopropyl)-carbodiimide. The method reduces inflammatory reactions of xenografts.		

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**TITLE****PREPARATION OF BIOLOGICAL MATERIAL FOR IMPLANTS****FIELD OF THE INVENTION**

5

The present invention relates to a method for the treatment of biological material for the use in implants, in which the implant has a reduced tendency for inflammatory, or other adverse reactions, compared to  
10 other treated biological material. In particular, the method relates to the preparation of tissue samples for use in medical devices intended for implant into a mammal.

**BACKGROUND OF THE INVENTION**

15

Numerous vascular prostheses have been produced for the replacement of mammalian arteries and veins. These include prostheses made from synthetic polymers, particularly Dacron™ polyester and Teflon™ fluoropolymers  
20 in both knitted and woven configurations as well as expanded polytetrafluoroethylene.

Biological vascular grafts have also been produced from arteries or veins, generally by methods that incorporate either proteolytic enzyme digestion followed  
25 by aldehyde fixation or aldehyde fixation alone. Other surface modifications have also been used. The objective of these methods was to stabilize tissues by crosslinking the proteinaceous components and without altering the thromboresistance properties of the vessels. Fixation  
30 with aldehydes tends to cause considerable alteration of mechanical properties of the vessel. Subsequent treatment with anionic detergents reduces the tendency to undergo calcification, as disclosed in U.S. 4,323,358. Other techniques to inhibit calcification have also been  
35 proposed.

U.S. 4,801,299 of K. Brendel et al. described methods which retained the extracellular matrix that provides the

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vessel with inherent mechanical properties, forms a highly integrated and dense network of crosslinked fibres and is essentially resistant to extraction by detergents and physiological solutions. In the method, a variety of  
5 tissues were extracted sequentially with non-ionic and ionic detergents to yield structures essentially free of cellular membranes, nucleic acid, lipids and cytoplasmic components.

U.S. 4,776,853 of P. Klement et al. describes an  
10 extraction procedure which provides a biological prosthesis that is equivalent in compliance and mechanical strength to a healthy vessel through retention of elastic properties and highly resistant to calcification and thrombogenesis. In the method, tissue is extracted by a  
15 series of detergent and non-proteolytic enzymatic treatments, to provide an acellular tissue.

The use of tissue-derived (bioprosthetic) materials requires chemical and physical pretreatment of tissues from other species (xenografts) prior to implantation as  
20 part of medical devices. The objectives of these treatments are: (i) to improve resistance to degradation by host cells and bacteria, primarily degradation by enzymatic means, (ii) to reduce immunological reactions and resulting degradation, and (iii) to sterilize the  
25 material. Treated bovine, porcine, ovine, and equine tissues are used in cardiovascular surgery e.g. in heart valve replacement, vascular grafting and patching applications.

Virtually all xenograft materials are currently  
30 treated with glutaraldehyde, primarily for crosslinking of collagen, but it is now understood that improved tissue treatments are needed. For instance, immunological recognition and reaction are not eliminated and new epitopes are formed. Furthermore, glutaraldehyde  
35 crosslinked tissue material is susceptible to calcification after implantation, and may produce local cytotoxicity in tissues surrounding the implant. This may

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be as a result of release of monomers following glutaraldehyde depolymerization and/or desorption. It is possible that polymerization of glutaraldehyde inhibits complete crosslinking of collagen by forming a polymeric  
5 coating on the surface of collagen fibres. Consequently, in the bioprosthesis industry, there is significant interest in seeking to achieve the objectives above without the associated disadvantages of glutaraldehyde, especially without inflammatory reactions.

10 U.S. 4 776 853 refers to cross-linking of collagen and elastin with glutaraldehyde, carbodiimide ( $\text{H}-\text{N}=\text{C}=\text{N}-\text{H}$ ) or polyglycerol polyglycidyl ether i.e. a polyepoxide. Carbodiimides are unique amongst this group and are known as "zero-length" crosslinkers, because they modify side-  
15 groups on proteins to make them reactive with other side-groups, so that a crosslink may be formed, but do not themselves remain in the linkage.

It is known, from E. Allaire et al, Journal of Vascular Surgery, p446-456 (1994), that chronic rejection  
20 of arterial xenografts results in arterial wall dilation and rupture, making them unsuitable for long-term arterial replacement in vascular surgery. The results obtained showed that all arterial xenografts, whether treated with sodium dodecyl sulfate or untreated, were aneurysmal two  
25 months after grafting.

#### SUMMARY OF THE INVENTION

A method for the treatment of biological material for  
30 use in implants to reduce inflammatory or other adverse reactions has now been found.

Accordingly, an aspect of the present invention provides a method for preparation of biological material for a medical device, comprising treating samples of  
35 intact or acellular tissue of such biological material in vitro with (i) a water-soluble carbodiimide of the formula  $\text{R}_1-\text{N}=\text{C}=\text{N}-\text{R}_2$ , wherein  $\text{R}_1$  and  $\text{R}_2$  are independently selected

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from alkyl and alkylamino groups, in combination with (ii) an agent that forms a stable activated ester therewith.

Another aspect of the invention provides a method for  
5 preparing biological material for implant in a mammal comprising the steps of:

(i) isolating from a suitable donor a tissue sample of said biological material;

(ii) treating said sample in vitro with (a) a  
10 water-soluble carbodiimide of the formula  $R_1-N=C=N-R_2$ , wherein  $R_1$  and  $R_2$  are independently selected from alkyl and alkylamino groups, in combination with (b) an agent that forms a stable activated ester therewith.

Another aspect of the invention provides in a method  
15 of forming an implant for a mammal from biological material, the improvement comprising treating samples of intact or acellular tissue of such biological material in vitro with (i) a water-soluble carbodiimide of the formula  $R_1-N=C=N-R_2$ , wherein  $R_1$  and  $R_2$  are independently selected  
20 from alkyl and alkylamino groups, in combination with (ii) an agent that forms a stable activated ester therewith.

A further aspect of the invention provides a method of reducing cytotoxic reactions to an implant formed from biological material, comprising treating samples of intact  
25 or acellular tissue of such biological material in vitro with (i) a water-soluble carbodiimide of the formula  $R_1-N=C=N-R_2$ , wherein  $R_1$  and  $R_2$  are independently selected from alkyl and alkylamino groups, in combination with (ii) an agent that forms a stable activated ester therewith.

30 In preferred embodiments of the method of the invention, the carbodiimide is 1-ethyl-3-(dimethylaminopropyl)-carbodiimide.

In a further embodiment, the agent to stabilize the activated esters is N-hydroxysuccinimide or N-  
35 hydroxysulfosuccinimide.

#### DETAILED DESCRIPTION OF THE INVENTION

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The present invention relates to a method for the treatment of biological material for use in a medical device that is intended for implant into a mammal, especially a human, or for use in veterinary applications.

It has been found that biological material treated in accordance with the invention and utilized in such implants offers advantages, especially with respect to inflammatory properties.

10 The biological material may be intact tissue or acellular tissue. The biological material is, or is derived from, normally healthy biological material that has been removed from a donor. The donors may include human, bovine, ovine, porcine, equine, canine, primate, 15 cervine and/or caprine donors. From such mammals, the biological material may be selected from any soft tissue, including for example hearts, arteries, veins, ureters, umbilical cords, skin, ligaments, tendons, cartilage, trachea, pericardium, placenta and the like.

20 Techniques for the selection, removal and preparation of the biological material prior to being subjected to the method of the invention are known e.g. as discussed by J.M. Lee in "Implants derived from tissues: Heart valves, patches and vascular grafts" Bureau of Medical Devices, 25 National Health and Welfare Canada (1990).

The biological material subjected to the method of the present invention may be intact tissue. The intact tissue may be subjected to the method substantially in the form in which it is obtained. It is to be understood that 30 such tissue would be washed with suitable physiological solutions e.g. Hanks solution, for the purpose of cleaning the sample prior to treatment. Extraneous material, such as adherent fat and loose connective tissue, should also be removed.

35 Techniques for the formation of acellular tissue are known in the art. For instance, P. Klement et al. describe, in U.S 4,776,853, a series of procedures for

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extraction and treatment of tissue in order to prepare an acellular tissue. Other extraction procedures may also be used, e.g. as discussed in the aforementioned U.S. patent of K. Brendel et al.

5       The biological material in the form of intact or acellular tissue is treated with a solution containing a carbodiimide and an agent that forms a stable activated ester. It is believed that the agent forms the stable ester with the biological material rather than with the  
10 carbodiimide per se. In general, the conditions of treatment e.g. temperature, pH and the like are similar to those used in other techniques for the treatment of tissue, whether intact or acellular tissue, and will be understood by persons skilled in the art of such  
15 treatments. Examples of such conditions are exemplified hereinafter.

The carbodiimide is a water-soluble carbodiimide of the formula  $R_1-N=C=N-R_2$ , wherein  $R_1$  and  $R_2$  are independently selected from alkyl and alkylamino. Preferably, the alkyl  
20 groups are lower alkyl, e.g.  $C_1-C_4$  alkyl. Examples of such alkyl groups are methyl, ethyl, propyl and butyl groups. The alkylamino groups include dialkylamino and dialkylamino alkyl groups, in which the alkyl group is preferably lower alkyl e.g.  $C_1-C_4$  alkyl. As the method is  
25 operated in aqueous solution, it is necessary for the carbodiimide to be soluble at an effective concentration in such solution, which is referred to herein as being water soluble. In a particularly preferred embodiment,  $R_1$  is ethyl and  $R_2$  is dimethylaminopropyl i.e. the  
30 carbodiimide is 1-ethyl-3-(dimethylaminopropyl) - carbodiimide (EDC). Other water-soluble carbodiimides may be used.

Agents that form the stable activated esters are known. In a preferred embodiment, the agent is N-  
35 hydroxysuccinimide (NHS) or N-hydroxysulfosuccinimide.

It will be appreciated that the treatment with EDC may be adversely affected by any buffer used in the

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treatment solution. For example, phosphate, Tris and acetate buffers may affect the treatment. Thus, it is understood that treatment will be effected in a compatible buffer, if a buffer is used.

5 As exemplified hereinafter, the treatment of biological material with cyanamide i.e. the carbodiimide of the formula given above in which  $R_1$  and  $R_2$  are each hydrogen, in the presence of, for instance N-hydroxysuccinimide, does not result in the effects and  
10 benefits of the method of the present invention.

The treated biological material is intended for use in medical devices for implant into humans. As discussed hereinafter, the treated tissue has been found to result in reduced immune recognition, reduced vascular  
15 inflammation, reduced foreign-body reaction, reduced degradation and elastin erosion, reduced mural thrombus, reduced perforations and mural erosions, and reduced perivascular inflammation. It is also believed that the treated tissue may result in reduced aneurysms.

20 The present invention is believed to be useful in the field of xenografts, and to provide improvements in such grafts. The grafts are particularly intended for use in mammals, especially humans. Nonetheless, such grafts may be used in mammals other than humans, for example in  
25 veterinary applications. It is further believed that the method of the invention will be useful in a wide variety of types of xenografts.

The present invention is illustrated by the following examples:

30 For the Examples I and II below, bovine hearts with intact pericardia were obtained from an abattoir within a few minutes of slaughter of 6-9 month old calves. The hearts were transported at ambient conditions in Hanks' physiological solutions to the test laboratory, where all  
35 adherent fat was gently stripped from the ventral surface of the pericardium. Suture markers were sewn into the pericardium near the base of the aorta and near the apex

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of the heart to indicate the base-to-apex direction. Rectangular sections of ventral pericardium, including the suture markers, were excised and washed in fresh Hanks' solution for 30 minutes (3 washes at 10 minutes each).

5

#### EXAMPLE I

A series of tests were conducted to illustrate the effect of variables in the method. In the tests, use of EDC and NHS illustrates the method of the invention.

10 Other tests are comparative tests.

In each series of tests, rectangular strips measuring 4 mm by 25 mm were cut from the excised ventral pericardial section of the bovine hearts. Fresh tissue strips were immediately tested for their thermal stability and served as controls. The remaining strips were subjected to one of the procedures below for treatment using EDC. During treatment, all solutions were constantly agitated. Tissue strips were removed from the treatment solutions after periods of 1 hour, 2 hours, 3 hours and 24 hours and in each case washed for 30 min. in 0.1M  $\text{Na}_2\text{HPO}_4$  (3 washes of 10 min. each). The tissue strips were evaluated by determining the collagen denaturation temperature,  $T_d$ , using the procedure of LEE J.M., PEREIRA C.A., ABDULLA D. et al., Med. Eng Phys. 17 (1995) 115-121.

25 The following tests were conducted:

(a) Effect of treatment time. In this test, the treatment solutions were unbuffered. Two identical 1.15% (w/w) EDC solutions were made using a molar ratio of 1:1 between EDC and the agent N-hydroxysuccinimide (NHS). An equal number of tissue strips were immersed into each solution; the results were normalized for sample size, but were essentially identical in this regard. During treatment, the solution was held constant at pH 5.5 by the addition of drops of 0.1M HCl or 0.1M NaOH solution, as required.

35

The results for constant pH are shown in Table I. In these results, and the results that follow, the mean value

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and standard error of the mean are reported.

(b) Effect of EDC concentration.

Equal numbers of tissue strips were immersed in EDC solutions (1:1 EDC:NHS, pH constant at 5.5) with 5 concentrations of 0.58%, 1.15% and 2.30% (w/w).

The results are shown in Table II.

(c) Effect of amount of NHS.

Equal numbers of tissue strips were immersed in 1.15% (w/w) EDC solutions with molar ratios of EDC:NHS of 10 1:2, 1:1, 2:1 and 4:1. The pH was held constant at 5.5.

The results are shown in Table III.

(d) Effect of Molecular Structure.

Equal numbers of tissue strips were immersed for 24 hours into equimolar solutions of EDC or cyanamide i.e. 15 1.15% EDC or 0.25% cyanamide solutions. Each solution had a molar ratio of EDC:NHS of 2:1 or of cyanamide:NHS of 2:1. pH was held constant at 5.5.

The results are shown in Table IV.

The results in this example illustrate the present 20 invention as well as illustrating effect of variables in the method of the present invention.

#### EXAMPLE II

Using procedures based on Example I, treatment of 25 bovine pericardium using EDC was compared with crosslinking using glutaraldehyde (GLUT) or cyanamide. Untreated tissue was used as a control.

The following parameters for EDC treatment of bovine pericardium were chosen: 1.15% EDC:NHS = 2:1, pH constant 30 at 5.5, room temperature.

Six cleaned and suture-marked ventral pericardia were washed in Hanks' physiological saline. From each pericardium, six rectangular pieces measuring 2 cm wide by 3 cm long were cut with their long sides parallel to the 35 base-to-apex direction. Each treatment group used two pieces, one for biochemical digestion tests and the other for mechanical and thermal stability tests. In order to

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reduce any effect of regional differences in the pericardium, each treatment group was assigned pieces from a different location with each subsequent pericardial sac.

Thus, with pericardia from six animals, each test group 5 within each treatment had samples from each of the six locations on the ventral pericardial surface.

The treatment groups were prepared as follows:

(1) Untreated (fresh) tissue was tested for its thermal stability and mechanical properties no later than 10 6 hours after slaughter. Fresh tissue for biochemical digestion was frozen in Hanks' solution and prepared as described below

(2) GLUT crosslinking was carried out according to the protocol described by Ionescu et al., J. Thorac. 15 Cardivasc. Surg. 73(1977) 31-42. The pericardial pieces were immersed in 0.5% GLUT (EM grade), buffered to pH 7.4 in 0.067 M phosphate buffer for 24 hours. The pieces were then removed and washed in 0.1 M  $\text{Na}_2\text{HPO}_4$  for 30 minutes (3 washes of 10 minutes). GLUT tissue for biochemical 20 digestion was frozen in Hanks' solution and prepared as described below.

(3) EDC treatment was carried out as follows:

An unbuffered 1.15% EDC (2:1 EDC:NHS) solution was made by dissolving 1.15 grams of EDC and 0.34 grams of NHS 25 in 100 ml of distilled water. The pH of the EDC solution was adjusted to 5.5 by the addition of 0.1 M HCl or 0.1 M NaOH solution. The pericardial pieces were immersed in the solution within one hour of solution preparation. The tissue remained in the EDC solution for 24 hours, and 30 throughout the fixation, the solution was maintained at pH 5.5. The pieces were then removed and washed in 0.1 M  $\text{Na}_2\text{HPO}_4$  for 30 minutes (3 washes of 10 minutes). EDC tissue for biochemical digestion was frozen in Hanks' solution and prepared as described below.

35 (4) Cyanamide treatment was carried out under the conditions described above for EDC treatment i.e. equimolar concentration of cyanamide were substituted for

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EDC. This treatment failed to increase the  $T_d$ , and so the samples were subjected to thermal stability testing only.

### Hydrothermal Stability Test

5 Tissue samples measuring 4 mm x 25 mm were mounted in a custom-built six-sample tester interfaced to a computer. The mounted specimens were immersed in a bath of distilled water at room temperature, loaded to 50 grams and held at constant extension. The bath was then heated  
10 to 95°C at a rate of approximately 2°C/min. The computer monitored the temperature of the bath and the loads on the six specimens, and recorded these measurements at 1°C increments. When the collagen denatured, the specimen contracted and a sharp increase in load was observed at  
15 the denaturation temperature. The procedures used are described by LEE J.M., PEREIRA C.A., ABDULLA D. et al., Med. Eng Phys. 17 (1995) 115-121.

### Biochemical Tests

20 Pericardial sections from all treatment groups were thawed and washed with several changes of distilled water for 2 hours (4 washes of 30 minutes each) in order to remove any excess treatment agents. Tissue sections were subsequently defatted (using 1:1 v/v chloroform:methanol,  
25 100% methanol, 50% methanol, and distilled water) and freeze dried. The freeze dried tissues were then minced and stored in the freezer.

Tests were conducted as follows:

(a) Enzymatic Degradation with Collagenase.

30 Samples (20-30 mg) of finely minced tissue were weighed and placed in Beckman polycarbonate ultracentrifuge tubes. A 5 mg/ml stock collagenase solution was prepared by dissolving bacterial collagenase (C. histolyticum, Sigma Type I) in 0.05 M Tris-HCl/10 mM  
35 CaCl<sub>2</sub> buffer (pH 7.4), to provide a final 0.5% (w/w) enzyme to tissue ratio in a final volume of 2-3 ml. Incubations

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were carried out at 37°C for 18 hours. At the end of the incubation period, samples were centrifuged for 20 minutes at 4°C. The supernatant was removed and the remaining pellets were washed with distilled water, re-centrifuged  
5 and freeze dried. The percentage of the initial mass remaining in the residue was used as a measure of resistance of the tissue to enzymatic degradation.

(b) Effect of Collagenase on Denatured Collagen.  
Tissue samples from different treatment groups were  
10 immersed in 0.05 M Tris-HCl/10 mM CaCl<sub>2</sub> buffer (pH 7.4) and heated in a 90°C heating block for 30 minutes to denature the collagen. Collagenase treatments were then carried out, as described above.

(c) Enzymatic Degradation with Trypsin.  
15 Conditions for trypsin digestion of denatured fresh tissue were established, and then applied to EDC-treated and GLUT-crosslinked bovine pericardia. Thus, trypsin was added to samples denatured as above at a 1:10 enzyme:tissue ratio (w/w), and the samples were then  
20 incubated in a 37°C shaking water bath for 48 hours. To determine the weight loss due to denaturation (loss of solubilized protein or other constituents), freeze dried pellets were again prepared as above, and the weights compared to the weights before denaturation.

25 (d) Chemical Degradation with CNBr.

Minced pericardia from different treatment group were accurately weighed (20-30 mg) and placed in Beckman polycarbonate ultracentrifuge tubes. The tubes containing tissue were then bubbled with N<sub>2</sub> for approximately 3  
30 minutes. A CNBr/formic acid (70% v/v) solution (25 mg/ml, previously flushed with N<sub>2</sub>) was then added to the tissue at a ratio of 0.1 ml per 10 mg tissue. The tubes were flushed with more N<sub>2</sub>, capped and agitated for 24 hours at room temperature. The solution was then diluted with 10  
35 volumes of distilled water, and the tubes were then left uncapped overnight. The next day, the percentage weight

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remaining in the pellet was determined as above.

(e) Amino Acid Analysis.

Pericardial samples from different treatment groups were hydrolyzed by a vapour phase/liquid phase reaction, using 6N HCl with 1% phenol at 110°C for 24 hour. After hydrolysis, the excess HCl was vacuum-dried and samples were redried using a solution of methanol:water:-triethylamine (2:2:1). The samples were then derivatized for 20 minutes at room temperature using methanol:water:-triethylamine:phenylisothiocyanate (7:1:1:1). The derivatized samples were dissolved in sample diluent and placed on a Waters PICO-TAG system. The amino acids were detected at 254 nm at 0.01 AUFS range.

**High Strain Rate Mechanical tests**

High strain rate mechanical tests were carried out generally using the method of Lee et al. ASTM Special Technical Publication 11: Biomaterials' Mechanical Properties (Kambic HE, Yokobori AT et. al.), Philadelphia. (1994) pp. 19-42. A tissue test sample measuring approximately 4 mm x 20 mm was cut from each of the rectangular sections with its long side parallel to the base-to-apex direction of the heart. In order to obtain a measure of sample thickness without disruption, the thickness of an adjacent piece of tissue was measured with a Mitutoyo non-rotating thickness gauge. The test sample was then mounted into brass grips such that the sample's grip-to-grip gauge length was approximately 10 mm. The upper grip was attached to a fixed load-cell and the lower grip was attached to the base of a plexiglass tank, containing Hanks' solution held constant at 37°C, located on Instron apparatus capable of sample displacement. In order to measure the test sample's actual gauge length, a small load of 0.5 grams was applied to the sample to remove all kinks, and the sample was photographed using a video camera. The test sample was then subjected to the following mechanical testing

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protocol:

Preconditioning: The sample was mechanically preconditioned by loading and unloading the sample between 5 0 grams and 80 grams at 1 Hz for 25 cycles using a triangular deformation waveform.

Stress-strain: The sample was loaded/unloaded, from 0 grams to 80 grams to 0 grams, using a single triangular 10 deformation waveform at 0.1 Hz. Load-extension data was collected. The sample was also tested at 1 Hz and 10 Hz with preconditioning before each test.

Stress relaxation: The sample was preconditioned and then 15 loaded from 0 grams to 40 grams in 0.1 seconds using a single ramp deformation waveform and held at constant extension for 100 seconds. Load-time data was collected. The sample was then preconditioned and the stress relaxation test repeated using an maximum load of 80 g.

20

Forced vibration: The sample was preconditioned and then loaded to 40 grams. A cyclic sinusoid waveform with a small amplitude of 0.05 mm was executed at 1 Hz and load-extension-time data were collected. The sample was also 25 cycled at 10 Hz about 40 grams, cycled at 1 Hz about 80 grams, and cycled at 10 Hz about 80 grams. Preconditioning was performed before each of these tests.

#### Analysis of Mechanical Data

30 The video image of each 0.5 gram-loaded test sample was used to calculate the dimensions of the sample. Using the sample's thickness, width and gauge length, load-deformation-time data was converted to stress-strain-time data, using procedures described by LEE J.M., HABERER 35 S.A., PEREIRA C.A. et al., in: ASTM Special Technical Publication 11: Biomaterials' Mechanical Properties (Kambic HE, Yokobori AT ed.), Philadelphia. (1994) pp. 19-

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42.

The results in this example illustrate the following:

(i) Processing Parameters for Carbodiimide Treatment

In the results obtained on the effect of controlling pH, effect of EDC concentration and effect of amount of NHS are reported in Tables I-III, respectively.

10

TABLE I Effect of Exposure Time					
Time hours	0	1	2	3	24
$T_d(^{\circ}\text{C})$	$68.4 \pm 0.3$	$73.4 \pm 0.4$	$75.7 \pm 0.5$	$76.7 \pm 0.3$	$76.4 \pm 0.3$

TABLE II Effect of EDC Concentration		
EDC Concentration	$T_d(^{\circ}\text{C})$ at 24 hours with NHS	$T_d(^{\circ}\text{C})$ at 24 hours without NHS
0.58%	$77.3 \pm 0.5$	$73.2 \pm 0.8$
1.15%	$76.4 \pm 0.3$	$75.0 \pm 0.0$
2.3%	$79.5 \pm 0.3$	$76.2 \pm 0.8$

TABLE III Effect of EDC:NHS Ratio				
EDC:NHS	1:2	1:1	2:1	4:1
$T_d(^{\circ}\text{C})$	$71.2 \pm 0.8$	$76.4 \pm 0.3$	$86.0 \pm 0.6$	$86.9 \pm 0.5$

15

Thermal denaturation studies of bovine pericardium treated with 1.15% EDC solution (EDC:NHS 1:1, pH 5.5 held

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constant) showed rapid changes within the first 3 hours, but no significant change in denaturation temperature ( $T_d$ ) thereafter (Table I). Without the pH being held constant during fixation, the solution became gradually more basic, reaching pH 6 after 24 hr. During this treatment,  $T_d$  values were slightly lower at 3 hours (compared to the pH-controlled experiments), but were not significantly different after 24 hours.

Denaturation temperature increased with EDC concentration, the 2.3% EDC solution (EDC:NHS = 1:1) showing greater final  $T_d$  than did 0.58% or 1.15% solutions (Table II). In the absence of NHS, the maximum  $T_d$  achieved was reduced. In addition, when the ratio of EDC:NHS was increased, greater treatment was achieved up to a maximum of 2:1; increasing the ratio to 4:1 had no significant effect (Table III).

(ii) Effect of Molecular Structure: Ineffectiveness of Cyanamide

The results obtained on effect of molecular structure are reported in Table IV.

TABLE IV			
Effect of Molecular Structure			
Denaturation Temperature	Fresh	EDC treated	Cyanamide treated
$T_d$	$69.7 \pm 1.2^\circ\text{C}$	$76.4 \pm 0.3^\circ\text{C}$	$68.5 \pm 0.3^\circ\text{C}$

When pericardium was treated in 1.15% cyanamide, with ratio of cyanamide: NHS at 2:1, pH 5.5, no increase in denaturation temperature was observed after up to 24 hours treatment. Denaturation temperature at 24 hours was  $68.5 \pm 0.3^\circ\text{C}$ , regarded as equivalent to the  $69.7 \pm 1.2^\circ\text{C}$  value for fresh tissue. No further characterization of the

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cyanamide-treated material was undertaken.

(iii) Comparison of EDC-Treated and Glutaraldehyde-Crosslinked Materials with Fresh Bovine Pericardium

5

The results of the above comparison are reported in Table V.

TABLE V				
Degradation of Treated Materials				
Material Treatment	Denaturation Temperature (°C)	Percent Mass Remaining after CNBr	Percent Mass Remaining after Collagenase	Percent Mass Remaining after Trypsin
Undenatured				
Fresh	$69.7 \pm 1.2$	$11.0 \pm 1.2$	$20.1 \pm 1.5$	---
Glutaraldehyde	$86.0 \pm 0.3$	$97.0 \pm 0.5$	$90.4 \pm 1.5$	---
EDC	$85.3 \pm 0.4$	$98.0 \pm 0.3$	$93.4 \pm 0.6$	---
Denatured				
Fresh	---	---	$9.7 \pm 0.6$	$5.8 \pm 0.6$
Glutaraldehyde	---	---	$77.3 \pm 0.7$	$36.3 \pm 0.8$
EDC	---	---	$81.8 \pm 0.9$	$83.2 \pm 0.4$

10 (a) Hydrothermal Stability

The denaturation temperature for fresh bovine pericardial tissue was  $69.7 \pm 1.2^\circ\text{C}$  (Table V). Both GLUT-crosslinked and EDC-treated tissues were more thermally stable than fresh tissue, with the results for the GLUT-crosslinked and EDC-treated tissues being similar.

15

(b) Biochemical Tests

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Collagenase Resistance: Under reagent conditions where fresh pericardial tissue had little resistance to attack by collagenase, both GLUT-crosslinked and EDC-treated tissues showed markedly higher resistance, with no significant difference in the resistance to collagenase between the two reagents. Hydrothermally denatured collagen was more susceptible to enzymatic degradation by collagenase. After denaturation, the EDC-treated tissue was slightly, but significantly, more resistant to collagenase than was GLUT-treated tissue.

(c) Trypsin Resistance

In the undenatured form, fresh pericardial tissue was highly resistant to attack by trypsin. After 48 hrs of incubation with trypsin at 4°C, fresh tissue had fully  $81.3 \pm 0.7$  % of its mass remaining, and  $70.8 \pm 0.2$  % when incubated at 37°C. There was no significant difference between trypsin digestion at 1:10 and 1:5 enzyme to tissue ratios. At 1:10,  $81.8 \pm 1.5$  % of the fresh tissue mass remained intact, compared to  $74.7 \pm 2.4$  % when the enzyme was available at twice the concentration.

Denaturation alone caused some weight loss in the pericardium due to solubility. Fresh samples were 97.4% insoluble, GLUT-crosslinked samples 99.9% insoluble, and EDC-treated samples 98.9% insoluble. The difference in solubilities between fresh and treated materials is significant.

Trypsin digestion was extremely effective against denatured fresh tissue. Treatment with EDC substantially inhibited digestion while GLUT crosslinking was significantly less effective in inhibiting digestion.

(d) CNBr Resistance

EDC treatment and GLUT crosslinking produced equivalent increases in resistance to solubilization by CNBr.

(e) Amino Acid Analysis

When amino acid analysis is used to assess GLUT crosslinking, the decrease in available lysine (Lys)

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residues is an indication of the extent of reaction, although not necessarily the extent of crosslinking. To normalize for the amount of collagen present, a ratio of lysine to leucine (Leu) that was not expected to react was used [RAMSHAW J.A., STEPHENS L.J., TULLOCH P.A., Biochim Biophys. Acta 1206 (1994) 225-230].

The Lys/Leu ratio was 0.86 in the fresh tissue. This ratio was much reduced in GLUT-crosslinked tissue (0.20), while it remained unchanged in EDC-treated (0.88) tissue. As with the unchanged lysine values, there was no change in the numbers of the aspartic or glutamic acid residues in EDC treated tissue. Although the links formed in the EDC reaction were hydrothermally stable (as shown in denaturation testing), they did not survive the acid hydrolysis used in the amino acid analysis, which is consistent with the formation of a peptide bond.

#### Mechanical Tests

The results of extensibility tests, expressed as strain at 200 kPa stress, are given in Table VI.

TABLE VI Extensibility Expressed as Strain (%) at 200 kPa Stress			
Frequency	Fresh	Glutaraldehyde	EDC
0.1 Hz	$3.1 \pm 0.5$	$9.0 \pm 0.8$	$12.3 \pm 1.0$
1 Hz	$3.0 \pm 0.5$	$8.7 \pm 0.9$	$12.1 \pm 0.8$
10 Hz	$2.2 \pm 0.4$	$5.6 \pm 0.8$	$8.5 \pm 0.8$

The stress-strain curves for GLUT-crosslinked or EDC-treated materials were similar. When strain under 200 kPa stress was examined, both GLUT-crosslinked and EDC-treated tissues were more extensible than fresh tissue, and EDC-treated tissue was more extensible than GLUT-crosslinked

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tissue.

The results of stress relaxation tests are given in Table VII.

TABLE VII Stress Relaxation Expressed as Per Cent Stress Remaining at 100 s			
Initial Load	Fresh	Glutaraldehyde	EDC
40 g	$52.2 \pm 3.0$	$63.8 \pm 1.0$	$76.5 \pm 1.7$
80 g	$57.2 \pm 2.6$	$68.4 \pm 1.9$	$75.3 \pm 1.2$

In stress relaxation tests, EDC-treated tissue displayed the least load decay (i.e. was most elastic) over a period of 100 seconds at fixed extension. The relaxation of the EDC-treated material was less than for both fresh and GLUT-crosslinked materials.

The results of the forced vibration tests are given in Table VIII.

10

TABLE VIII Magnitude of Dynamic Modulus (MPa)				
Material	40 g; 1 Hz	40 g; 10 Hz	80 g; 1 Hz	80 g; 10 Hz
Fresh	$27.8 \pm 4.0$	$30.8 \pm 4.2$	$40.2 \pm 5.6$	$44.8 \pm 6.4$
Glut.	$15.4 \pm 2.2$	$14.7 \pm 1.2$	$22.4 \pm 1.9$	$24.0 \pm 1.9$
EDC	$6.7 \pm 0.8$	$7.2 \pm 0.9$	$12.3 \pm 1.4$	$13.9 \pm 1.5$
Phase Angle (degrees)				
Material	40 g; 1 Hz	40 g; 10 Hz	80 g; 1 Hz	80 g; 10 Hz
Fresh	$1.6 \pm 0.6$	$1.3 \pm 0.5$	$0.7 \pm 0.3$	$2.5 \pm 1.4$
Glut.	$1.1 \pm 0.5$	$2.0 \pm 1.1$	$1.3 \pm 0.5$	$1.7 \pm 0.8$
EDC	$2.1 \pm 0.4$	$0.2 \pm 0.2$	$1.6 \pm 0.5$	$0.6 \pm 0.5$

The lower stiffness of the EDC-treated and GLUT-

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cross-linked materials at a given stress level was confirmed in forced vibration tests. For both the 40 gram and 80 gram mean load levels, and at both 1 Hz and 10 Hz cycling, EDC-treated tissue had the lowest magnitude of dynamic modulus, and fresh tissue the highest. The phase angles for all three tissue groups were each below 5° i.e. the materials were quite elastic in small vibrations.

### EXAMPLE III

10 Each of four acellular matrix processed caprine carotid arteries that had been treated according to the procedures of the aforementioned patent of Klement et al. and that had been stored in phosphate buffer solution (PBS) was cut into 4 sections having a length of 5-6 cm.  
15 The sections were subjected to one of the following treatments:

a) Control

Control samples were kept in PBS at 4°C.

b) NHS treatment

20 Sections were treated with N-hydroxysuccinimide (NHS) at a concentration of 0.34 g/100 ml, without addition of EDC or cyanamide, for 24 hrs. The pH of the solution was monitored for the first two hours and maintained at a pH of 5.5.

25 c) Treatment with EDC:NHS mixture

Sections were treated with a 1.15% EDC solution (admixed with NHS in a 2:1 molar ratio) for a period of 24 hours. The pH was monitored for the first two hours and maintained at 5.5.

30 d) Treatment with Cyanamide:NHS mixture

Sections were treated with a 1.15% solution of cyanamide (admixed with NHS in a 2:1 molar ratio) for a period of 24 hours. The pH was monitored for the first two hours and maintained at 5.5.

35 The sections thus obtained were cut into two portions, one of which was subjected to Denaturation Temperature Testing using the procedure described above

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and the other subjected to biochemical testing.

The section subjected to biochemical testing was defatted using chloroform/methanol solution, 100% methanol, 50% methanol, 50% methanol, and water. The defatted tissue was freeze-dried and weighed. It was then minced and heated to 90°C in water for denaturation of collagen. Trypsin was added at a 1:10 enzyme to tissue ratio. The samples were then incubated at 37°C for 48 hrs, in a shaking water bath, and centrifuged for 20 minutes at 30,000 rpm. For each sample, the solubilized fraction was removed, while the remaining pellet was freeze-dried. The percentage of initial mass remaining in the residue was determined, being a measure of the resistance of tissue to enzymatic digestion.

The results obtained, reported as mean  $\pm$  standard deviation, were as follows:

TABLE IX Effect of Treatments on Trypsin Digestion of Undenatured Materials		
Material* Treatment	Percent Mass Remaining after Trypsin	Denaturation Temperature ( $T_d$ )
Control	21.4 $\pm$ 6.1	57.5 $\pm$ 1.6
NHS	22.1 $\pm$ 6.9	62.5 $\pm$ 1.2
Cyanamide + NHS	19.5 $\pm$ 2.2	62.5 $\pm$ 1.2
EDC + NHS	83.5 $\pm$ 10.4	82.5 $\pm$ 1.0

\* NHS = N-hydroxy succinimide

EDC = 1-ethyl-3-(dimethylaminopropyl) carbodiimide

The results show that treatment with cyanamide viz. the carbodiimide of the formula  $R_1-N=C=N-R_2$ , wherein  $R_1$  and  $R_2$  are each hydrogen, in the presence of an agent defined herein viz. N-hydroxysuccinimide, had no effect on the denaturation temperature of the sample, which is

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consistent with the results obtained in Example II. Moreover, such treatment resulted in a tissue that underwent extensive enzymatic degradation. Such results were essentially identical to the result obtained in the  
5 untreated control sample and the sample that had been treated with only N-hydroxysuccinimide. In contrast, the samples treated according to the invention, using EDC:NHS, showed a relatively small amount of enzymatic degradation and a denaturation temperature substantially higher than  
10 the control sample, thereby showing effects of treatment according to the method of the invention.

#### EXAMPLE IV

Samples of tissue were treated and tested for  
15 invasion of inflammatory cells in vivo.

Twenty (20) caprine and twelve (12) canine carotid arteries were processed to acellular matrix vascular grafts using the technique of the aforementioned patent of Klement et al. Eight caprine acellular grafts were  
20 further treated with a solution of 15 g/L of 1-ethyl-3-(3-dimethyl-aminopropyl)-carbodiimide hydrochloride (EDC) and 3.5 g/L of N-hydroxysuccinimide (NHS) in a phosphate buffer at pH 5.5 for 24 hours. All the grafts were then washed three times in phosphate buffer saline and once in  
25 water before being sealed in a container containing 1% propylene oxide in 50% aqueous ethanol.

Twelve (12) non-EDC treated caprine grafts and twelve (12) non-EDC treated canine grafts (autogenous saphenous vein grafts) were implanted into twelve (12) dogs  
30 bilaterally in the carotid position. The eight (8) caprine EDC-treated grafts were implanted bilaterally as interpositional grafts in the femoral arteries of 4 dogs (2 per animal).

The results of histological analysis are given in  
35 Table X.

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TABLE X			
Histological Finding	Frequency		
	Canine non-EDC autografts	Caprine non-EDC xenografts	Caprine EDC xenografts
patency	12/12	1/12	8/8
immune inflammatory cells in graft wall	0/12	12/12	0/8
mural thrombus	0/12	10/12	2/8
perforations/mural erosions	0/12	9/12	0/8
foreign-body giant cells in graft walls	0/12	11/12	0/8
matrix degradation/elastin erosion	0/12	9/12	0/8
perigraft inflammation	0/12	9/9*	0/8

\* could only be determined in 9 of 12 cases.

5

The results are discussed below.

#### EXAMPLE V

The use of treatment with glutaraldehyde to prevent invasion of inflammatory cells *in vivo* was tested.

Caprine and canine carotid arteries were processed to acellular matrix vascular grafts using the technique of the aforementioned patent of Klement et al. The caprine acellular grafts were further treated with a solution of 0.2 to 2% glutaraldehyde. These grafts were then washed in phosphate buffer saline and sterilized using 25 k Greys of high energy irradiation process.

Six caprine xenografts and six canine allografts were implanted as interpositional grafts in the femoral arteries of dogs for 90 days. None of the allografts were occluded at explant while four of the six glutaraldehyde-treated xenografts were occluded at explant.

The results of histological analysis of the glutaraldehyde-treated xenografts are given in Table XI.

25

Table XI		
Histological Finding	Frequency	
	Canine non-GLUT allografts	Caprine GLUT xenografts
patency	6/6	2/6
immune cells in graft wall	0/6	0/6
mural thrombus	0/6	4/6
perforations/mural erosions	0/6	0/6
inflammatory cells in graft	0/6	0/6
perigraft inflammation	0/6	6/6
perigraft foreign-body giant cells in graft wall	0/6	6/6
matrix degradation/elastin erosion	0/6	0/6
mesenchymal cells in media	6/6	0/6
mesenchymal cells in adventitia	6/6	0/6

5

EXAMPLE VI

The use of treatments with glutaraldehyde or polyepoxides to prevent invasion of inflammatory cells *in vivo* was tested.

10 Canine carotid arteries were processed to acellular matrix vascular grafts using the technique of the aforementioned patent of Klement et al. The acellular grafts were further treated with a solution of glutaraldehyde or polyepoxides using the technique of  
 15 (Courtman D.W., Lee J.M., Yeger H., Wilson G.J. (1992) Adv. Biomater. 10:241-246). These grafts were then washed in phosphate buffer saline and implanted subcutaneously in rats.

After 30 days, the samples were removed. The results  
 20 of histological examination of the samples are given in Table XII.

TABLE XII			
Histological Finding	Frequency		
	Epoxide ACM	GLUT ACM	CONTROL (NONGLUT) ACM
Mononuclear inflammatory cells in graft media (mean $\pm$ SEM)	$0.06 \pm 0.03$ cells/ $10^4 \mu\text{m}^2$	0 cells/ $10^4 \mu\text{m}^2$	$2.6 \pm 0.8$ cells/ $10^4 \mu\text{m}^2$
Perigraft foreign body inflammatory response with macrophages and multinucleated giant cells	moderate	intense	minimal

The results obtained are discussed below.

5 The EDC treatment reduced immune recognition and vascular inflammation as compared to xenografts without EDC treatment. This is shown by the reduction in immune inflammatory cells in the graft wall from a frequency of 12/12 without EDC treatment versus a frequency of 0/8 with  
10 EDC treatment (Table X).

Mural thrombosis was also reduced from a frequency of 10/12 without EDC treatment to a frequency of 2/8 with EDC treatment. This was also a reduction compared to the 4/6 frequency observed with GLUT treatment (Table X).

15 EDC treatment also reduced perforations and mural erosions from a frequency of 9/12 without EDC treatment to the frequency of 0/8 with EDC treatment (Table X).

A comparison of perivascular inflammation demonstrated an improvement with EDC treatment (a  
20 frequency of 0/8) over no treatment (a frequency of 6/6) and over GLUT treatment (a frequency of 6/6) and poly epoxide treatment.

The EDC treatment reduced the foreign-body reaction as shown by the reduction of giant cells and macrophages  
25 in the graft wall from a frequency of 11/12 without EDC treatment to a frequency of 0/8 with EDC treatment (Table X). This was also true for the perigraft region which

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contained substantial numbers of foreign-body giant cells with GLUT and polyepoxide treatments but not with EDC treatment.

In addition, the EDC treatment resulted in reduced  
5 degradation and elastin erosion from a frequency of 9/12 without EDC treatment to a frequency of 0/8 with EDC treatment (Table X).

The lack of erosion of the elastin after EDC  
treatment suggests that the vascular grafts prepared with  
10 EDC would be less likely to form aneurysms or perforations over extended periods of time.

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**CLAIMS:**

1. A method for preparation of biological material for a medical device, comprising treating samples of  
5 intact or acellular tissue of such biological material in vitro with (i) a water-soluble carbodiimide of the formula  $R_1-N=C=N-R_2$ , wherein  $R_1$  and  $R_2$  are independently selected from alkyl and alkylamino groups, in combination with (ii) an agent that forms a stable activated ester with said  
10 carbodiimide.
2. The method of Claim 1 in which the sample is treated in a solution of a compatible buffer.
- 15 3. The method of Claim 2 in which the agent that forms the stable activated ester is N-hydroxysuccinimide.
4. The method of Claim 2 in which the agent that forms the stable activated ester is N-  
20 hydroxysulfosuccinimide.
5. The method of any one of Claims 1-4 in which the carbodiimide is 1-ethyl-3-(dimethylaminopropyl)-  
25 carbodiimide.
6. The method of any one of Claims 1-4 in which the tissue is intact tissue.
7. The method of any one of Claims 1-4 in which the  
30 tissue is acellular tissue.
8. The method of Claim 1 in which the carbodiimide is 1-ethyl-3-(dimethylaminopropyl)-carbodiimide and the agent that forms the stable activated ester is N-  
35 hydroxysulfosuccinimide.

- 30 -

9. The method of any one of Claims 1-4 and 8 in which said suitable donor is human, bovine, ovine, porcine, canine, primate, cervine or caprine.

5 10. The method of any one of Claims 1-4 and 8 in which the biological material is selected from hearts, arteries, veins, ureters, umbilical cords, skin, ligaments, tendons, cartilage, trachea, pericardium and placenta.

10

11. A method for preparing biological material for implant in a mammal comprising the steps of:

(i) isolating from a suitable donor a tissue sample of said biological material; and

15 (ii) treating said sample in vitro with (a) a water-soluble carbodiimide of the formula  $R_1-N=C=N-R_2$ , wherein  $R_1$  and  $R_2$  are independently selected from alkyl and alkylamino groups, in combination with (b) an agent that forms a stable activated ester.

20

12. The method of Claim 11 in which the sample is treated in a solution of a compatible buffer.

13. The method of Claim 11 in which the agent that  
25 forms the stable activated ester is N-hydroxysuccinimide.

14. The method of Claim 11 in which the agent that forms the stable activated ester is N-hydroxysulfosuccinimide.

30

15. The method of any one of Claims 11-14 in which the carbodiimide is 1-ethyl-3-(dimethylaminopropyl)-carbodiimide.

35 16. The method of any one of Claims 11-14 in which the tissue is intact tissue.

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17. The method of any one of Claims 11-14 in which the tissue is acellular tissue.

18. The method of Claim 11 in which the carbodiimide is 1-ethyl-3-(dimethylaminopropyl)-carbodiimide and the agent that forms the stable activated ester is N-hydroxysulfosuccinimide.

19. The method of any one of Claims 11-14 and 18 in which said suitable donor is human, bovine, ovine, porcine, canine, primate, cervine or caprine.

20. The method of any one of Claims 11-14 and 18 in which the biological material is selected from hearts, arteries, veins, ureters, umbilical cords, skin, ligaments, tendons, cartilage, trachea, pericardium and placenta.

21. In a method of forming an implant for a mammal from biological material, the improvement comprising treating samples of intact or acellular tissue of such biological material in vitro with (i) a water-soluble carbodiimide of the formula  $R_1-N=C=N-R_2$ , wherein  $R_1$  and  $R_2$  are independently selected from alkyl and alkylamino groups, in combination with (ii) an agent that forms a stable activated ester with said carbodiimide.

22. The method of Claim 21 in which the sample is treated in a solution of a compatible buffer.

23. The method of Claim 21 in which the agent that forms the stable activated ester is N-hydroxysuccinimide.

24. The method of Claim 21 in which the agent that forms the stable activated ester is N-hydroxysulfosuccinimide.

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25. The method of any one of Claims 21-24 in which the carbodiimide is 1-ethyl-3-(dimethylaminopropyl)-carbodiimide.

5        26. The method of any one of Claims 21-24 in which the tissue is intact tissue.

27. The method of any one of Claims 21-24 in which the tissue is acellular tissue.

10

28. A method of reducing inflammatory reactions to an implant formed from biological material, comprising treating samples of intact or acellular tissue of such biological material in vitro with (i) a water-soluble  
15 carbodiimide of the formula  $R_1-N=C=N-R_2$ , wherein  $R_1$  and  $R_2$  are independently selected from alkyl and alkylamino groups, in combination with (ii) an agent that forms a stable activated ester with said carbodiimide.

20        29. The method of Claim 28 in which the sample is treated in a solution of a compatible buffer.

30. The method of Claim 29 in which the agent that forms the stable activated ester is N-hydroxysuccinimide.

25

31. The method of Claim 29 in which the agent that forms the stable activated ester is N-hydroxysulfosuccinimide.

30        32. The method of any one of Claims 28-31 in which the carbodiimide is 1-ethyl-3-(dimethylaminopropyl)-carbodiimide.

33. The method of any one of Claims 28-31 in which  
35 the tissue is intact tissue.

34. The method of any one of Claims 28-31 in which

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the tissue is acellular tissue.

35. The method of Claim 28 in which the carbodiimide is 1-ethyl-3-(dimethylaminopropyl)-carbodiimide and the  
5 agent that forms the stable activated ester is N-hydroxysulfosuccinimide.

36. The method of any one of Claims 28-31 and 35 in which said suitable donor is human, bovine, ovine,  
10 porcine, canine, primate, cervine or caprine.

37. The method of any one of Claims 28-31 and 35 in which the biological material is selected from hearts, arteries, veins, ureters, umbilical cords, skin,  
15 ligaments, tendons, cartilage, trachea, pericardium and placenta.

38. The method of any one of Claims 11, 21 and 28 in which the implant is a xenograft.  
20

39. The method of any one of Claims 11, 21 and 28 in which the implant is an allograft.

## INTERNATIONAL SEARCH REPORT

In' ional Application No  
PCT/CA 97/00056

A. CLASSIFICATION OF SUBJECT MATTER  
IPC 6 A61L27/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)  
IPC 6 A61L

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 95 22361 A (BIOMEDICAL DESIGN INC ;GIRARDOT JEAN MARIE (US); GIRARDOT MARIE NA) 24 August 1995 see page 7, line 1 - line 15 see page 9, line 23 - line 35 see page 10, line 15 - line 25 ---	1-29
A	EP 0 121 008 A (NIMNI MARCEL E) 10 October 1984 ---	
A	WO 90 09102 A (BAXTER INT) 23 August 1990 -----	

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

\* Special categories of cited documents :

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# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No

PCT/CA 97/00056

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